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Characteristics of cadmium tolerance in ‘Hermes’ flax seedlings: Contribution of cell walls

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Most flax (*Linum usitatissimum*) varieties are described as tolerant to high concentrations of Cd. The aim of the present paper was to better characterize this tolerance, by studying the responses of flax plantlets, cv Hermes, to 18 d growth on 0.5 mM Cd. In Cd-treated seedlings, the majority of Cd was compartmentalized in the roots. Analysis of other elements showed that only Fe concentration was reduced, while Mn increased. Growth parameters of Cd treated flax were only moderately altered, with similar mass tolerance-indices for roots and shoots. Tissue anatomy was unaffected by treatment. The effect on lipid per-oxidation, protein carbonylation and antioxidative activities appeared low but slightly higher in roots. The most important impacts of Cd were, in all organs, cell expansion, cell-wall thickening, pectin cross-linking and increase of cell-wall enzymatic activities (pectin methylesterase and peroxidase). Thus, the role of the cell wall in Cd tolerance might be important at two levels: (i) in the reinforcement of the tissue cohesion and (ii) in the sequestration of Cd.

1. Introduction

Cadmium is a major environmental pollutant, with concentrations that can reach 200 mg kg⁻¹ in soil (e.g. Dahmani-Muller et al., 2000). At the whole-plant level, the major symptom of Cd toxicity is a global decrease in growth rate (An, 2004). At the organ level, browning and enlargement of roots are often reported (Barceló and Poschenrieder, 1990), while in leaves, chlorosis, stunting, and rolling are often observed (Sanità di Toppi and Gabbriellini, 1999). Within cells, Cd toxicity is associated with oxidative stress (Sandalo et al., 2001), membrane damage, and alteration of numerous enzymatic activities (Békésiová et al., 2007).

Some plant species exhibit resistance or tolerance to heavy metals, in varying degrees (Clemens, 2006). One resistance strategy is to avoid metal absorption by the roots. The avoidance mechanisms include reduction of absorbant hair surface and secretion of organic acids or mucilages into the rhizosphere. Another resistance strategy is cation compartmentalization in the root either by restricting them in cortical tissues, or by decreasing the vessel number and diameter. Within the cell, several strategies are used to maintain cellular metabolism such as exclusion at the surface of plasmalemma, sequestration in vacuole, complexation via metal-chelating molecules in cytosol and immobilization in cell wall

(CW) (Clemens et al., 2002). Indeed, the CW is the first barrier to toxic metals in the environment (Wang et al., 2008). Immobilization of Cd in the CW of maize (Lozano-Rodriguez et al., 1997) and lettuce (Ramos et al., 2002) has been demonstrated to contribute to Cd tolerance in these species.

Flax can accumulate high levels of Cd and is considered as a tolerant species. Shi and Cai (2009) reported that flax plants cultivated in the presence of 50–200 mg Cd kg⁻¹ soil exhibited a limited reduction of growth. The authors concluded that flax has an innate resistance to Cd stress and is moderately tolerant (tolerance index in the range of 40–62%).

In our laboratory growth conditions, we also observed that ‘Hermes’ seedlings could tolerate high concentrations of Cd, up to 0.5 mM, with little variation of the mass of the hypocotyl (Douchiche et al., 2007). At this concentration, the organization of the seedling tissues is unaffected by Cd, however, the metal provokes a reorientation of the hypocotyl growth axis, i.e. a reduction in elongation and an enhancement of cell expansion. These structural alterations are associated with a significant increase of the thickness of the cell walls, accompanied by a variation in uronic acids originating from pectin remodelling and an increase in their cross-linking. Similar to our previous report (Douchiche et al., 2007), the present study was conducted with 0.5 mM Cd, but at 10 and 18 days, in order to investigate the growth response, the Cd content and the level of antioxidative activities of all organs. These two growth stages are representative of the development of primary tissues (10 d) and tissues differentiating secondary wall

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(18 d). Particular attention was paid to pectin content (through the level of uronic acid) and the activity of CW remodelling enzymes. Altogether, the data provide new insights into mechanisms of Cd tolerance.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of flax (cv 'Hermes', a gift of the Cooperative Terre de Lin, France) were sown on culture medium containing either 2.6 mM $\text{Ca}(\text{NO}_3)_2$ or 0.5 mM $\text{Cd}(\text{NO}_3)_2$ and 2.1 mM $\text{Ca}(\text{NO}_3)_2$, as previously reported (Douchiche et al., 2007). Roots, hypocotyls and epicotyls (including cotyledons) were excised from plantlets collected after 10 or 18 d (10 d and 18 d respectively). All plantlets whose hypocotyl length was less than 1 cm were not collected to avoid analysing plants experiencing extreme toxicity. Three independent culture experiments were run.

2.2. Histological study

Hand cross-sections of root were stained with carmine-green as previously described (Brutch et al., 2008) and observed with a light microscope (Leica microsystem, Austria).

2.3. Cadmium and cation contents

After digestion of dried samples with $\text{HNO}_3/\text{HClO}_4$ (3/1; v/v), the content of Cd and cations (Ca, Mg, Na, K, Mn, Fe) in the organs were measured by atomic absorption spectrometry (Analyst 300, Perkin-Elmer, USA).

2.4. Antioxidative enzyme activities

Fresh tissue was ground in 100 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA, 1% (w/v) polyvinylpyrrolidone and 1 mM phenylmethanesulfonyl fluoride. Homogenate was centrifuged (12 000g, 20 min, 4 °C) for protein (Bradford, 1976) and enzyme activity measurements.

Activities of superoxide dismutase (SOD, E.C. 1.15.1.1), catalase (CAT, E.C. 1.11.1.6) and ascorbate peroxidase (APX, E.C. 1.11.1.11) were assayed as described by Qiu et al. (2008). Guaiacol peroxidase (GPX, E.C. 1.11.1.7) activity was measured as described by Paynel et al. (2009). Activities were expressed as nkatal g^{-1} FW, except for SOD (U g^{-1} FW).

2.5. Lipid peroxidation and protein-bound carbonyl content

Lipid peroxidation was determined by measuring the concentration of thiobarbituric acid-reacting substances (TBARS) as described by Buege and Aust (1972) and was expressed as nmol TBARS g^{-1} FW.

Carbonyl groups were assayed using the dinitrophenyl hydrazine method, according to Levine et al. (1991). Carbonyl content was calculated using an extinction coefficient for aliphatic hydrazones of 22 000 $\text{M}^{-1} \text{cm}^{-1}$ and was expressed as nmol g^{-1} FW.

2.6. Extraction of cell-wall uronic acids

Fresh tissue was ground in liquid nitrogen. The powder was treated in organic solvents for the isolation of CW. The CW yield was the mass (g) of dry CW per 100 g of fresh organ. Then, the CW was successively treated with trifluoroacetic acid (2 M TFA, 110 °C 2 h) and sulfuric acid (concentrated H_2SO_4 , 25 °C 1 h, followed by a dilution to 1 M, 110 °C 2 h) (Douchiche et al., 2007).

The two filtrates (TFA and H_2SO_4) were kept aside for uronic acid (UA) analyses (Blumenkrantz and Asboe-Hansen, 1973).

2.7. Cell-wall remodelling activities

Fresh tissue was ground in liquid nitrogen, and the ionically-bound proteins were obtained as previously described (Paynel et al., 2009): CW pectin methylesterase (CW PME, E.C. 3.1.1.11) activity was measured using the alcohol oxidase method and CW peroxidase (CW GPX, E.C. 1.11.1.7) activity was determined using guaiacol as substrate.

3. Results

3.1. Impact of cadmium on growth and anatomy

The elongation of flax plantlets, cultivated on 0.5 mM Cd was significantly reduced, especially in the root (Fig. 1A). The biomass and the mass ratio between root and shoot were moderately affected, mainly because the growth axis was reoriented. The mass tolerance-index ($\text{TI} = 100 \times [\text{biomass in Cd}] / [\text{biomass in control}]$) was high, in the range of 79–84%. Also, the water content of all organs remained constant.

Cd did not have an impact on root histological organization, but the root diameter was increased mainly due to the bigger cortical cells (Fig. 1B). More secondary roots were counted per section, mainly due to the length reduction of the primary root. The carmine-green staining of the rhizodermis, endodermis and xylem appeared more contrasted. Xylem was more expanded and exhibited four poles (Fig. 1B4) compared to the two poles in control roots (Fig. 1B3). The expansion of the xylem into four poles started as soon as 10 d (data not shown). This would correspond to an acceleration of the differentiation of tissues with secondary wall.

3.2. Cadmium accumulation and its effect on cation content

In treated plantlets, Cd concentration was about four times higher in roots than in shoots and was equivalent in hypocotyl and epicotyl (Table 1). Cd content in roots increased with time but did not vary much in shoots. When expressed as amount per plant, 3.2 μg of Cd was accumulated in root compared to 1.7 μg in shoot, at 18 d.

Among the ions studied, Na and K were the main ones accumulated in all organs. After 18 d of treatment, the concentration of Na decreased specifically in root while that of Mg increased in shoot. Cd also impacted other micronutrients, with a marked and opposite variation of Fe (decrease) and Mn (increase) content in all organs.

3.3. Effect of cadmium on lipid peroxidation, carbonyl content and antioxidative enzyme activities

Cd treatment had only a limited effect on lipid peroxidation and carbonylation of proteins. TBARS content increased only in 10 d Cd-exposed root and in 18 d Cd-exposed epicotyl (Fig. 2A). No significant difference in soluble protein level was observed between treated and control organs, except for an increase in protein observed in 18 d Cd-treated roots (Fig. 2B). Within these organs, the amount of oxidatively modified amino acids accounted for only about 10 ppm (Fig. 2C).

The impact of Cd on the activity of antioxidative enzymes (*i.e.* SOD, CAT, APX and GPX, Fig. 2D–G) was insignificant in most organs. At 10 d, only root APX displayed a significantly higher activity level than control. At 18 d, only CAT activity was significantly increased in Cd-treated roots, while in treated epicotyls

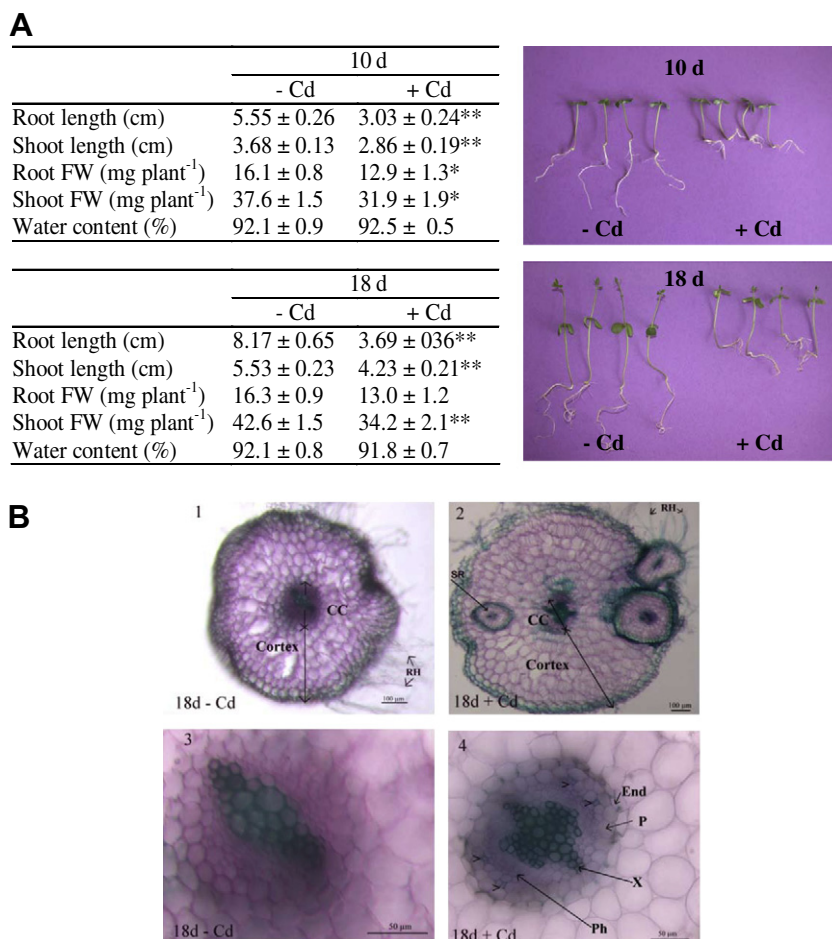


Fig. 1. Effect of cadmium on the growth of flax plantlets (A) and root anatomy (B). Seedlings were grown in the absence (–) or presence (+) of cadmium and harvested at 10 and 18 d (10 d and 18 d, respectively). Values represented means ± SE ($n = 30$). * and ** indicated significant differences between –Cd and +Cd, at $p < 0.05$ and $p < 0.01$, respectively. Microscopic observations of hand-cut sections were carried out on root proximal parts of 18 d seedlings, after coloration with carmine-green. B1 and B3: in the absence of Cd, B2 and B4: in the presence of Cd. B1 and B2: whole root section; B3 and B4: zoom on the central cylinder area. CC: central cylinder; Cortex: rhizodermis and cortical parenchyma; End: endodermis; FW: fresh weight, P: pericycle; Ph: phloem; RH: root hairs; SR: secondary root; X: xylem. Arrowheads: sclerified fibres. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Effect of cadmium on the cation content. Seedlings were grown in the absence (–) or presence (+) of cadmium and harvested at 10 and 18 d (10 d and 18 d, respectively). The results were expressed as $\mu\text{mol g}^{-1}$ dry weight (DW). E: epicotyl; H: hypocotyl; R: root. Values represented means of three replicates ($\text{SE} \leq 15\%$).

$\mu\text{mol g}^{-1}$ DW	10 d			18 d		
	–Cd			+Cd		
	R	H	E	R	H	E
Cd	0	0	0	20.0	5.4	7.0
K	963	1346	432	1130	1072	469
Na	659	218	76	567	249	88
Mg	165	97	438	100	113	461
Ca	104	136	85	131	146	130
Fe	4.80	0.56	3.11	0.36	0.28	0.88
Mn	0.40	0.48	1.30	0.31	1.38	3.04

SOD activity decreased and GPX activity increased. The GPX activity possibly compensated for the observed SOD decrease.

Altogether, the variations of lipid peroxidation between organs in presence and absence of Cd were correlated to those of APX activity ($r^2 = 0.94$).

3.4. Impact of cadmium treatment on the cell-wall yield and uronic acid content

To assess the effect of Cd on pectin metabolism, we analysed the CW yield and CW-UA content (Table 2). At 10 d, CW yield increased

in root and hypocotyl following Cd treatment. This increase resembled the age-dependent CW yield increase observed in untreated tissues and might be related to the acceleration of xylem differentiation described above (see Fig. 1).

Concerning UA, they were assayed firstly in TFA (hydrolysing matrix polysaccharides) and designated as TFA-UA, and secondly in H_2SO_4 (hydrolysing the residual sugars) and named H_2SO_4 -UA. According to Douchiche et al. (2007), TFA-UA were more extractable and consisted of rhamnogalacturonan (RG) structure while H_2SO_4 -UA would instead have originated from homogalacturonan (HGA) that was either associated

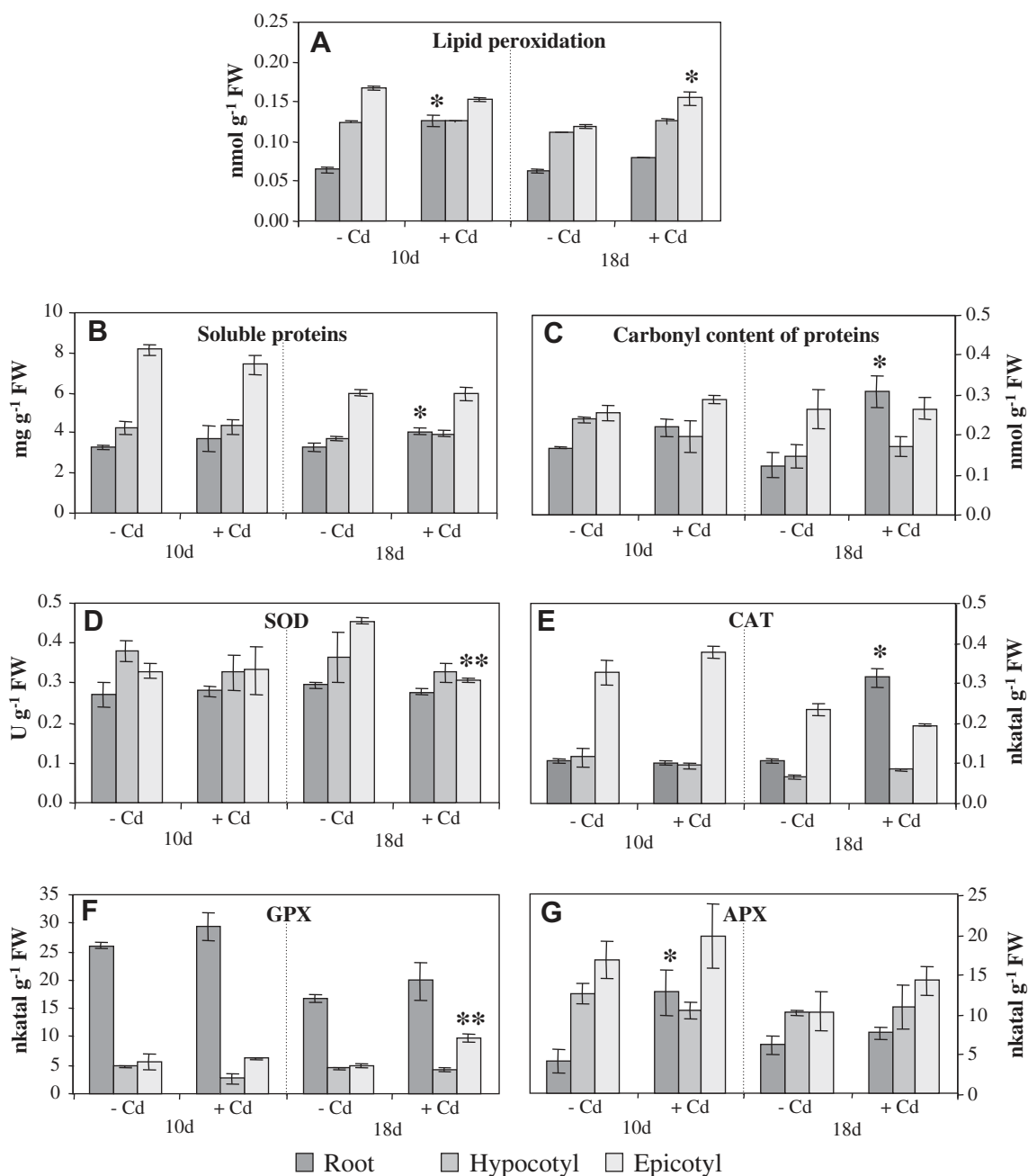


Fig. 2. Effect of cadmium on lipid peroxidation (A), soluble protein content (B), carbonyl content of proteins (C), SOD (D), CAT (E), GPX (F) and APX (G) *in vitro* activities. Seedlings were grown in the absence (–) or presence (+) of cadmium and harvested at 10 and 18 d (10 d and 18 d, respectively). Values represented mean \pm SE ($n = 3$). * and ** indicated significant differences between –Cd and +Cd, at $p < 0.05$ and $p < 0.01$, respectively.

Table 2

Effect of cadmium on the cell-wall yield and on the extractability of uronic acids. Seedlings were grown in the absence (–) or presence (+) of cadmium and harvested at 10 and 18 d (10 d and 18 d, respectively). Cell-wall (CW) yield was the mass (g) of dry CW per 100 g of fresh organ. Uronic acids (UA) were extracted by TFA, followed by H_2SO_4 and expressed as $mg\ g^{-1}$ CW dry weight. CW yield was the average of three experiments ($SE < 10\%$). UA values represented means of five independent CW samples ($SE \leq 10\%$).

	10 d						18 d					
	–Cd			+Cd			–Cd			+Cd		
	R	H	E	R	H	E	R	H	E	R	H	E
CW yield (%)	4.1	3.4	5.5	5.6	3.9	5.4	4.8	4.2	4.5	5.6	4.2	5.2
UA (TFA + H_2SO_4)	130	148	110	121	170	117	147	184	145	157	182	141
UA-TFA	84	74	77	68	75	59	83	81	94	104	95	83
UA- H_2SO_4	46	74	33	53	95	58	64	102	51	52	86	58

with cellulose or precipitated beside cellulose during TFA treatment.

The UA amount released in TFA was generally larger than in H_2SO_4 (except in Cd-treated hypocotyl), indicating a high level of

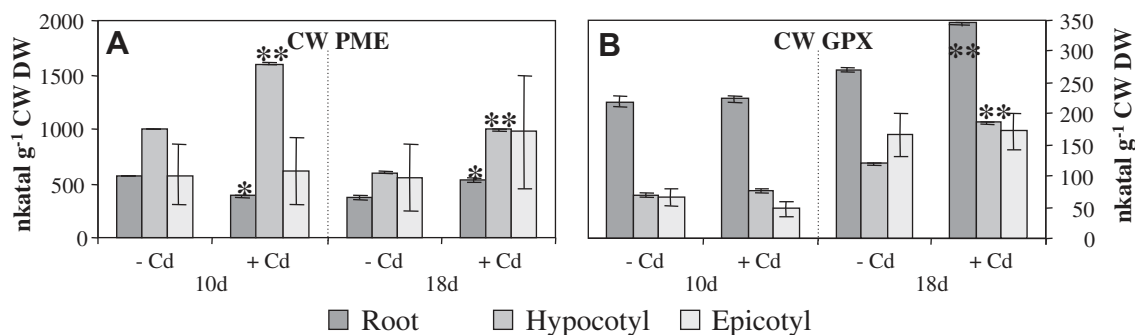


Fig. 3. Effect of cadmium on cell-wall ionically-bound pectin methylesterase (CW PME) (A) and guaiacol peroxidase (CW GPX) (B) *in vitro* activities. Seedlings were grown in the absence (–) or presence (+) of cadmium and harvested at 10 and 18 d (10 d and 18 d, respectively). Values represented mean \pm SE ($n = 3$). * and ** indicated significant differences between –Cd and +Cd, at $p < 0.05$ and $p < 0.01$, respectively.

RG in CW matrix. In 10 d treated plantlets, the TFA-UA level decreased whereas the UA amount released in H_2SO_4 was enhanced. At 18 d, the main Cd impact led to an increase of TFA-UA.

Globally, the total UA amount (TFA + H_2SO_4) was not affected by Cd, but increased with age from 135 ± 12 to 160 ± 10 mg g⁻¹ CW. Such values corresponded to a maximum cation exchange capacity (CEC) of 675–800 μ eq g⁻¹ CW. These CEC values would be high enough to neutralize the moderate absorbed amount of Cd (estimated for 12–60 μ eq g⁻¹ CW).

3.5. Effect of cadmium on cell-wall pectin methylesterase and peroxidase

The effect of Cd on CW PME activity was maximal in hypocotyl with an increase of 60% and 67% at 10 d and 18 d, respectively (Fig. 3A). In root, the CW PME activity decreased slightly at 10 d while it increased at 18 d. In epicotyl, only a statistically insignificant increase of PME activity was observed at 18 d.

Compared to CW PME activity, the effect of Cd on CW GPX activity was observed later (at 18 d), with an increased activity in both root and hypocotyl (Fig. 3B). These data suggested that the cross-linking of pectins described in hypocotyl (Douchiche et al., 2007) might also occur in root. Although the CW GPX activity increased with age in epicotyl, no impact of Cd was observed whatever the age.

4. Discussion

4.1. Flax 'Hermes' is a variety tolerant to cadmium

Plant growth inhibition is commonly used as an indicator of Cd toxicity. In flax, the main impact of Cd treatment was reorientation of the growth axis of the hypocotyl (Douchiche et al., 2007) and of the root (the present study). The tolerance index was in agreement with a recent study that demonstrated the Cd tolerance capacity ($59\% < TI < 72\%$) of another flax cultivar (Shi and Cai, 2009). Moreover, Cd had no visible impact on organ anatomy. Hence, based on plant growth response, 'Hermes' seedlings appeared tolerant to high Cd concentration. Because the CW contributes greatly to the determination of the growth axis of organs, we discuss next whether CW yield and UA content might be related to Cd tolerance.

Some studies have shown that the CW is subject to structural modulations following abiotic stress (e.g. McCann et al., 1994). Our data showed that at 10 d, Cd induced an increase of H_2SO_4 -UA in all organs. We suggest that cells, in order to adapt the plant to Cd stress, have reinforced the cell junctions *via* HGA acidification and subsequently *via* an increase of calcium pectates. These moieties would not be hydrolysed by TFA but would be detected in

H_2SO_4 . Such an hypothesis has been validated in hypocotyl where the HGA acidification was shown to be catalysed by a specific CW-PME isoform named B2, which was only present at 10 d (Paynel et al., 2009). Also, this acidification was related with the increase of HGA immunogoldlabelling in cell junctions (Douchiche et al., 2010). In root and epicotyl, such an increase of B2 would be accompanied by a decrease of one or more forms. Incidentally, the activity decrease in root could be correlated to the decrease of TFA-UA.

At 18 d, the Cd-induced increase of matrix-UA was concomitant with an increase of CW-PME and most importantly of CW-GPX activities. We suggest that, after the reinforcement of cell junctions at 10 d, cells would increase the cross-linking of the pectic matrix within the walls. This would allow an increased resistance to cell swelling observed in both root and hypocotyl. To our knowledge, only a few papers (e.g. Konno et al., 2005) have reported an impact of metal on pectin cross-linking.

Other general indicators of metal tolerance include cation absorption and antioxidative capacity. Cd had been shown to interfere with uptake, transport and use of several ions (Das et al., 1997). Our investigations showed that Cd did not have a general negative effect on cation composition, possibly because of the observed acceleration of xylem differentiation in the root and the keeping of the water uptake. However, the inhibition of Fe absorption we observed suggested that Cd might be carried into cells by Fe transporters. Although this inhibition provoked the reduction of Fe in shoots, we did not observe damages on the leaves which might be related to the enhancement of Mn observed in all organs. In stressed plants, chlorosis of leaves is among the first symptoms of Cd toxicity, resulting from the deficiency of Fe (Siedleska and Krupa, 1999) and/or Mn (Goldbold and Huttermann, 1985). Baszynski et al. (1980) demonstrated a partial restoration of tomato chloroplast structure damaged by Cd when Mn was added. So, in 'Hermes' seedlings, the Fe decrease seemed to be compensated by the Mn increase. Together with the increase of Mg occurring in the shoot, our data suggest no or low impact of Cd on photosynthesis, as reported by Shi and Cai (2009) who showed no effect of Cd on chlorophyll content of flax.

Measurements of carbonyl group and TBARS content allow to assess protein oxidation and lipid peroxidation and consequently to estimate oxidative stress. Increase of TBARS content in Cd-treated plants is often observed, even in hyperaccumulators (Qiu et al., 2008). In 'Hermes' seedlings, lipid peroxidation took place transiently in root and epicotyl while some increase of protein oxidation occurred only in root. This indicated that Cd did not induce severe oxidative stress in 'Hermes' plantlets. Moreover, the slight increase of soluble proteins observed in root was an indicator that senescence was not induced (Costa and Spitz, 1997).

Reactive oxygen species generated by Cd are counteracted by antioxidative enzymes such as SOD, CAT, APX and GPX. The

significant variations of the enzyme activities only happened when lipid peroxidation or protein carbonylation occurred. In root, our results indicated that APX and CAT functioned successively as the main H₂O₂ quenchers. In epicotyl, the main quencher was GPX. Thus, complementary antioxidative mechanisms are used depending on the organ and the age, as reported in Cd-treated *Arabis paniculata* (Qiu et al., 2008).

4.2. Tolerance of 'Hermes' plantlets would be related to cadmium compartmentalization strategy

Compartmentalization of Cd in roots contributes to tolerance at the whole-plant level (Ernst et al., 1992). In 'Hermes' seedlings, Cd was accumulated more in roots than in shoots, which is in agreement with previous reports for other flax varieties (Angelova et al., 2004; Shi and Cai, 2009). As suggested by Clemens et al. (2002), Cd compartmentalization in 'Hermes' root may be related to the enhanced suberization and lignification observed in the endodermis that might act as a barrier against the diffusion of Cd and translocation to the shoots. Also, we observed that cells (mainly cortical ones) became voluminous in root suggesting that vacuolation might increase to sequester the metal (Verbruggen et al., 2009).

In addition, tolerance may also depend on compartmentalization within the CW, although this mechanism remains controversial. Many studies on Al toxicity have shown that Al resistance is correlated with low binding to the CW (Eticha et al., 2005). These authors found that a low pectin content and/or a high degree of methylesterification of pectin characterised the roots of Al-resistance genotypes. Thus, exclusion mechanism plays a major role in Al-resistance. In contrast, other studies have described the key role of the CW in Cd neutralization and hence in Cd tolerance in plants (Uraguchi et al., 2009), including hyperaccumulators (Cosio et al., 2005). In roots and leaves of white lupin for example, the amount of Cd fixed by CW would be twice more than the amount of Cd complexed with thiol-compounds (Vázquez et al., 2006). Thus, the capacity of CW to bind Cd represents a protective action against the deleterious effect of this metal by reducing its cytosolic amount.

None of the previous studies of flax had highlighted the role of the CW in the fixation of Cd. Preliminary data on 'Hermes' seedlings indicated that a large amount of Cd accumulated in organs was retained in the CW (data not shown). Although artefactual adsorption of cations onto the negative charges of CW UA may occur, these data suggested that the CW could contribute to reduced entrance of Cd into the cytosol. Importantly, the present study indicates that the root CW-CEC was large enough to neutralize the totality of the absorbed Cd. This is also interesting in light of our previous studies of flax hypocotyl where we demonstrated (Douchiche et al., 2007, 2010) a compartmentalization of low-methylesterified HGA in the outer domain of CW that would keep Cd away from the vicinity of the plasmalemma.

5. Conclusion

Our study demonstrates the Cd tolerance of 'Hermes' seedlings: Cd has little effect on biomass, tissue organization, antioxidative enzymes and cation distribution. 'Hermes' tolerance was related to an adaptation of growth and a reinforcement of tissue cohesion via CW-pectin cross-linking. Interestingly, the main CW characteristics described in the stem were also observed in the root. To tolerate Cd, this variety developed a root compartmentalization strategy, and metal sequestration into CW that has to be further investigated by the localization of Cd.

Our data also showed that the shoot Cd concentration (660 mg Cd kg⁻¹ DW) was significantly higher than the threshold (100 mg Cd kg⁻¹ DW) defined by Baker et al. (2000) for Cd hyperaccumulators, underlining the possible use of 'Hermes' in phytoremediation. However, to conclude on the phytoremediation capacity of 'Hermes', experiments have to be conducted on mature plants grown in polluted field, as described by Dahmani-Muller et al. (2000).

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